ELSEVIER

Brain Research 793 (1998) 1–6

BRAIN RESEARCH

Research report

Adenoviral gene transfer to spinal cord neurons: intrathecal vs. intraparenchymal administration

A.J. Mannes [a], R.M. Caudle [a], B.C. O'Connell [b], M.J. Iadarola [a,*]

[a] *PNMB / NIDR / NIH, Bethesda, MD 20892, USA*
[b] *GTTB / NIDR / NIH, Bethesda, MD 20892, USA*

Accepted 18 November 1997

Abstract

The spinal cord is the site of many chronic, debilitating, neurological disorders that may be amenable to gene therapy. The present study, using quantitative and anatomical methods, examines the ability of replication deficient adenovirus to transfer a transcription cassette composed of the cytomegalovirus promoter driving the expression of the *LacZ* reporter gene (AdCMVβgal) to spinal-cord neurons. Rats were microinjected with AdCMVβgal into the spinal-cord parenchyma or subarachnoid space and sacrificed between 1 and 60 days post-infusion. The spinal cord was assayed for β-galactosidase (β-gal) activity fluorometrically (MUG). Intraparenchymal injection resulted in significant β-gal activity at day 1, which peaked at day 7, and decreased at day 14 (21-, 57- and 9.8-fold of control respectively). The spatial distribution of β-gal activity on day 7 was confined to the 1-cm section containing the injection site but was detected 2 cm caudal to this section by day 14. Histochemical staining and immunocytochemistry revealed a prominent reaction product in neurons, particularly motor neurons, and glia within the ventral grey matter bilaterally. Intrathecal viral injections showed comparatively modest, yet significant increases in β-gal activity throughout the spinal cord with the greatest activity (170% control) closest to the catheter tip. This study demonstrates that AdCMVβgal injected into the ventral spinal cord results in extensive in vivo neuronal gene transfer with β-gal activity reaching a peak by day 7 and remaining detectable at 60 days. Intrathecal viral injections result in greater spatial distribution but a comparatively lower level of expression.

Keywords: Adenovirus; Gene therapy; β-Galactosidase; Spinal cord; Motor neurons; Subarachnoid; MUG; Central nervous system

1. Introduction

The spinal cord is an essential site for both sensory and motor function. It coordinates the complex interactions of afferent sensory and efferent motor pathways via neurotransmitters, receptors and second messenger systems. Inborn or acquired aberration of these interactions can result in profound neurologic disorders ranging from chronic pain to degenerative motor disease. The use of various gene-transfer methodologies offers a novel approach to manipulate specific, localized biochemical interactions with the potential to ameliorate these disease processes. Several methods have been investigated to place genetic material in spinal-cord neurons. These include peripheral injection of adenovirus in muscle [13], retrograde axonal transmission of plasmids via peripheral nerve [23], fibroblast grafting [15] and direct parenchymal injections [18] with various degrees of success. In comparing these techniques, viral vectors appeared to provide the most efficient gene transfer.

Of the viral vectors currently being explored for gene transfer (herpes simplex virus, adenovirus, adeno-associated virus, retrovirus), adenovirus offers substantial advantages. Adenovirus can be raised to high titers, does not require a helper virus, can be rendered replication deficient, functions independently of cell replication, is non-oncogenic, and has been used without complications in vaccines [8]. Adenoviral DNA does not integrate into the host genome, which removes the complication of interrupting an important host gene, but may limit its ability to confer long-term expression [4]. Previous work in brain and spinal-cord tissue has shown that it is neurotrophic, but the spectrum of cellular recognition is quite broad [9,18]. For a review of viral vectors used in gene transfer see Ref. [25].

* Corresponding author. Bld. 49/Rm. 1A11, NIDR, NIH, 49 Convent Drive MSC 4410, Bethesda, MD 20892-4410, USA. Fax: +1-301-402-0667; E-mail: iadarola@yoda.nidr.nih.gov

0006-8993/98/$19.00

The present study compares two techniques for transferring genes to the rat spinal cord: intrathecal and intraparenchymal injections. Using a replication deficient adenovirus containing the cytomegalovirus (CMV) promoter and *LacZ* reporter gene, these routes of administration were examined with histochemical, immunocytochemical and quantitative biochemical analyses.

2. Materials and methods

2.1. Adenovirus

The adenovirus used for the current project, AdCMVβgal was replication deficient and included the CMV promoter and *LacZ* reporter gene. Briefly, a plasmid containing the Ad5 genome (pJM 17) was cotransfected in HEK 293 (human embryonic kidney) cells with a plasmid containing the CMV (cytomegalovirus) immediate early promoter and *LacZ* reporter gene. Through homologous recombination, an E1A$^-$ (deleted) viral vector was formed. This replication deficient virus was plaque-purified, grown in 293 cells and concentrated/purified to a titer of 4×10^{10} plaque-forming units (pfu)/ml. A complete description of the virus construction can be found in Ref. [3].

2.2. Intrathecal and parenchymal injections

All experiments were approved by the NIDR Animal Care and Use Committee (ACUC), and NIH Biosafety Committee and procedures were conducted in accordance with the IASP standards for the treatment of animals [29]. Male Sprague–Dawley rats (Harlan), 350–400 g, were anesthetized with an i.p. injection of a 1:1 mixture of ketamine (100 mg/ml) and Rompun (xylazine 20 mg/ml, Miles, Shawnee Mission, KS) in a volume of 1 ml/kg. The spinal cord was exposed at the level of the first cervical vertebra and a 27-gauge Whitacre needle, which has a sharp solid point with the orifice at the side of the tip, was advanced 0.5 mm from the midline and 2.5 mm from the surface. Five microliters of AdCMVβgal (1×10^{10} pfu/ml) were infused at 1 μl/min. The needle was left in place for an additional minute, withdrawn and the incision closed with 3-0 vicryl sutures. Upon recovery from anesthesia, animals were returned to their home cages and allowed food and water ad libitum.

A complete description of the intrathecal catheter placement and injections can be found in Ref. [28]. Rats, 350-400 g, were anesthetized as above and the spinal cord exposed at the first cervical vertebra. A catheter made from PE-10 tubing (Intramedic, Parsippany, NJ) was advanced 8 cm into the intrathecal space and 5 μl of AdCMVβgal (1×10^{10} pfu/ml) was infused at a rate of 1 μl/min. One minute post infusion, the catheter was removed and the incision closed. The animals were allowed to recover and returned to their cages.

2.3. β-Gal MUG assay

Rats were euthanized with CO_2, the entire spinal cord removed and the meninges separated. The spinal cord was sectioned into 1-cm segments (the first centimeter section was centered on the injection site) and frozen. Tissue was assayed for β-gal enzyme activity fluorometrically with 4-methylumbelliferyl β-D-galactoside (MUG) as described [19]. The fluorometric assay was adapted for use with a 96-well plate reader with minor modifications. Tissue samples were weighed and homogenized with a polytron (Brinkman, Westbury, NY) in 0.75 ml Z-Buffer (60 mM $Na_2PO_4 \cdot H_2O$; 40 mM $NaH_2PO_4 \cdot H_2O$; 10 mM KCl; 1 mM $MgSO_4 \cdot 7H_2O$; pH = 7.0). Each well contained 1 mg tissue, 30 μl MUG (3 mM) and 50 μl Z-buffer. The plate was incubated at 37°C for 1 h, 75 μl stop solution (300 mM glycine; 15 mM EDTA; pH = 11.2) was added to each well and the plate was read. Preliminary studies with this assay showed linearity over time (out to 2 h) and the tissue concentrations utilized. A standard curve was generated using *E. coli* β-galactosidase (Sigma, St. Louis, MO). MUG assay results are reported as enzyme units/mg tissue (1 E.U. will hydrolyze 1.0 μmole of *O*-nitrophenyl β-D-galactoside/min at pH 7.3, at 37°C). Each tissue sample was assayed in triplicate and averaged. Significance was determined by ANOVA and Neuman–Keuls post-hoc analysis; criterion for significance was $P < 0.05$.

2.4. Histochemical and immunocytochemical analysis

Rats were anesthetized with 1 ml/kg of a 1:1 mixture of ketamine (100 mg/ml) and xylazine (Rompun, 20 mg/ml), perfused with 100 ml cold phosphate-buffered saline (pH = 7.4) and 250 ml 4% paraformaldehyde, the spinal cord was removed and post-fixed in 4% paraformaldehyde overnight and transferred to 30% sucrose for 24 h. The tissue was sectioned at 40 μm, incubated in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (x-gal, 1 mg/ml final concentration from a 20 mg/ml stock solution in *N,N*-dimethyl formamide) and buffer (100 mM sodium phosphate, 1.3 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$ for 3 h at 37°C and mounted on slides. At pH 7.3, the activity of the bacterial enzyme is distinguished from the mammalian endogenous, lysosomal β-gal-like activity [19,24]. For immunocytochemistry, rats were perfused as above and the spinal-cord tissue sectioned at 30 μm, incubated with a rabbit antibody against *E. coli* β-galactosidase (1:200 dilution; 5-Prime 3-Prime, Boulder, CO), further processed using the Vectastain ABC Kit (Vector, Burlingame, CA) and mounted on slides.

3. Results

3.1. Parenchymal injections

The spinal-cord injections at the cervical level resulted in high expression of β-gal activity. β-gal activity (4.22 $\pm$

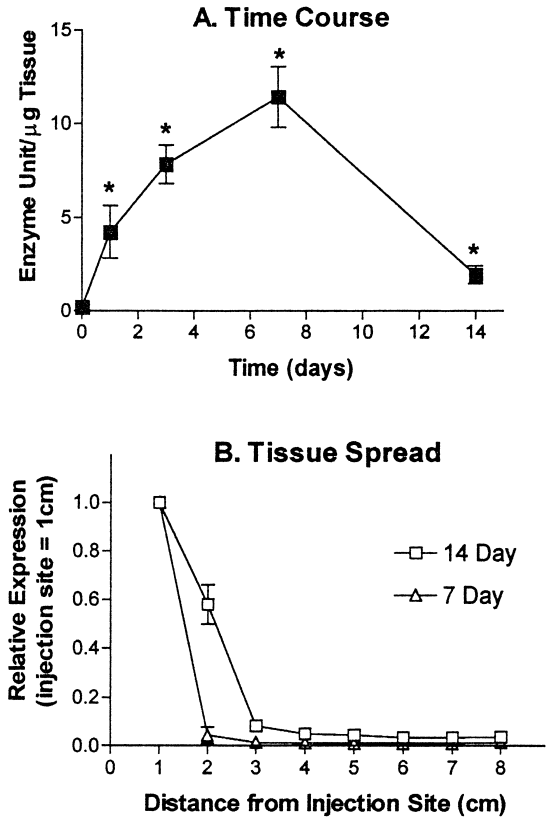

Fig. 1. Intraparenchymal injections. Expression of β-galactosidase activity in the rat spinal cord at various times (A) and spatial distribution (B) after injection with 5 μl AdCMVβgal (1×10^{10} pfu/ml). The spinal cord was cut into 1-cm sections, with the first section centered on the injection site, and enzyme activity was quantified using the fluorescent MUG assay. Samples were run in triplicate and averaged ($n=3$ rats/time point). Significance ($*\ P<0.05$) was determined using ANOVA and Neuman–Keuls post-hoc test. (A) Enzyme activity of the 1-cm spinal cord section containing the injection site assayed at 0, 1, 3, 7, 14 days. (B) Spatial distribution of the enzyme activity in contiguous 1-cm sections of spinal-cord tissue at 7 and 14 days. The activity is shown as a ratio of the maximal expression for each time point. Enzyme levels were normalized to show relative spatial distribution of enzyme activity in the spinal cord.

1.41 E.U./μg) was significantly elevated (21-fold) over non-injected control tissue (0.198 ± 0.005 E.U./μg) by one day after injection. Activity increased to 11.44 ± 1.63 E.U./μg (57-fold) by day 7 and remained elevated (1.95 ± 0.484 E.U./μg, 9.8-fold) at 14 days. Significant activity above control (0.235 ± 0.008 E.U./μg) was still detectable at day 60 (Fig. 1A). Assay of the 1-cm spinal-cord sections successively further from the injection site showed a time-dependent spread of enzyme activity. At days 1, 3, and 7, activity was largely confined to the 1-cm section containing the injection site. By 14 days, the overall level of expression had diminished from 57-fold (peak) to a 10-fold elevation at the site of injection but extended caudally into the two adjacent segments which showed a 5.6- and 0.8-fold elevation respectively (Fig. 1B). No significant elevation in activity was detected in the remaining spinal-cord sections.

3.2. Intrathecal injections

In animals in which the catheter tip was located at the sacral level and given a single intrathecal injection, enzyme activity in the spinal cord at 7 days was 0.292 ± 0.031, 0.227 ± 0.018, 0.234 ± 0.018, 0.195 ± 0.016 E.U./μg for the sacral, lumbar, thoracic and cervical regions respectively as compared to the tissue blank (0.172 ± 0.008 E.U./μg). The activity in sacral, lumbar and thoracic sections was statistically significant as noted in Fig. 2A. The highest elevation was in the sacral region at the approximate tip of the catheter. The pia mater MUG value of 0.792 ± 0.087 E.U./μg represented a statistically significant fivefold increase above the blank value (Fig. 2B, $P<0.05$).

3.3. Histochemical and immunocytochemical analysis

Histochemical staining of the cervical spinal cord from rats sacrificed 7 days post injection demonstrated a high level of β-galactosidase activity. Both glial cells and neurons exhibited enzyme activity with the most robust accumulation of reaction product in cells in the anterior grey matter (Fig. 3). Although the needle was placed in a

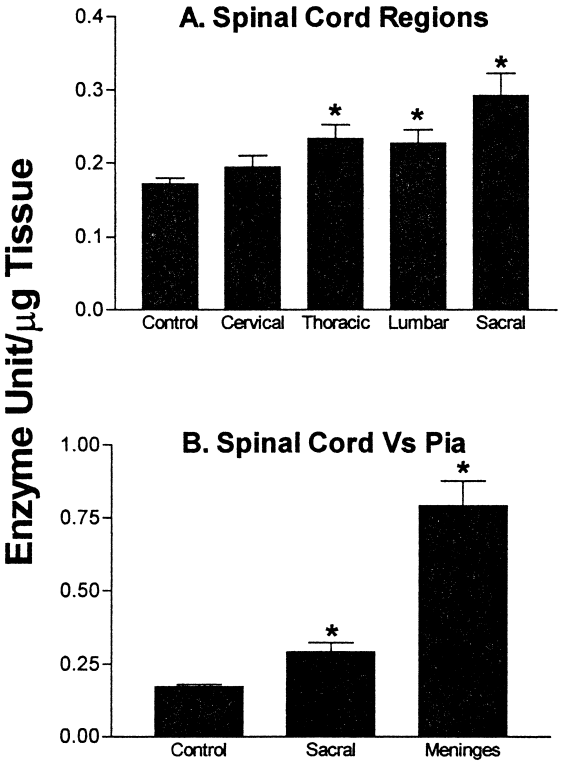

Fig. 2. Intrathecal injections. Distribution of β-galactosidase activity in the rat spinal cord at 7 days after intrathecal injection with 5 μl AdCMVβgal in the sacral region. The spinal cord was removed and cut into cervical, thoracic, lumbar and sacral regions and enzyme activity quantified by MUG assay. The meninges was removed and processed separately. (A) Expression shown for spinal levels ($*\ P<0.05$). (B) Relative expression of meninges to spinal cord tissue in the sacral region.

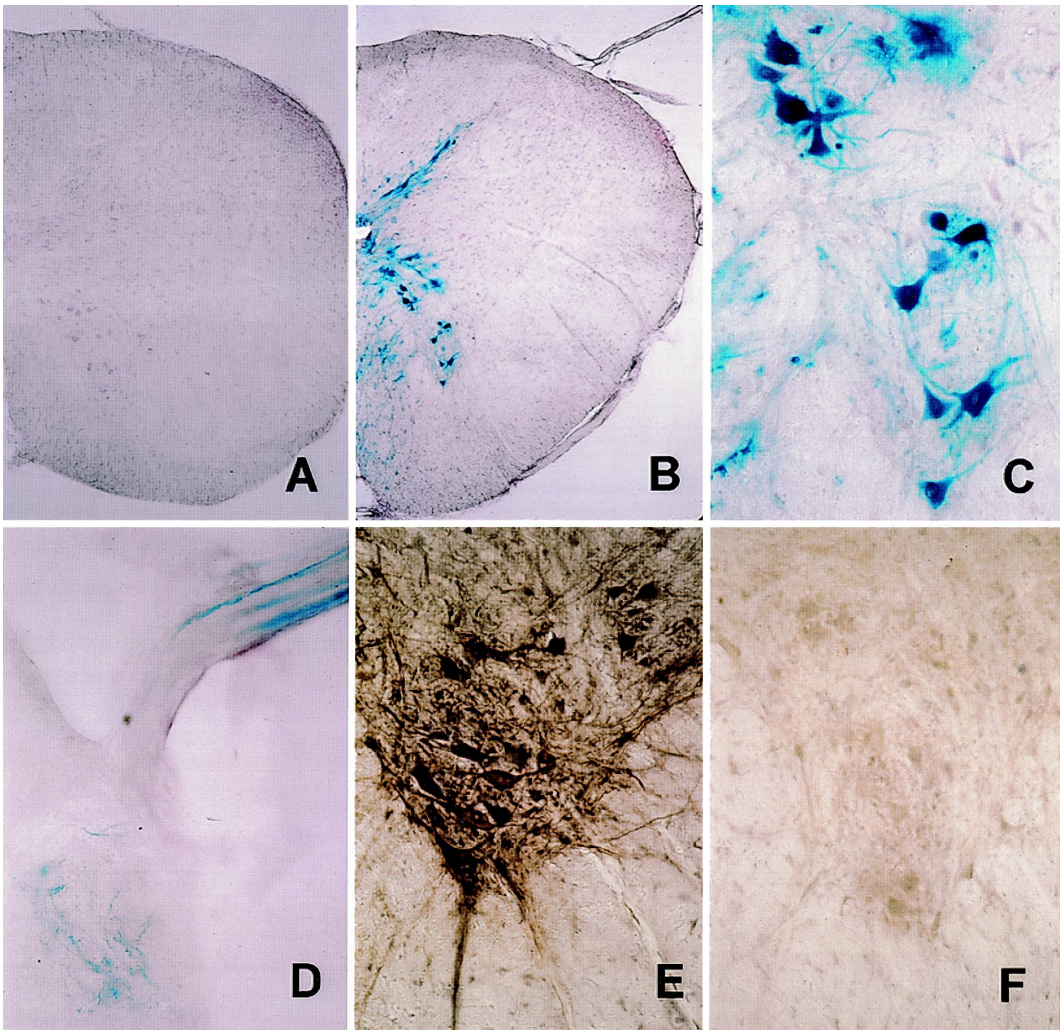

Fig. 3. Histochemical staining and immunocytochemistry. Rat spinal cord was injected with 5 μl AdCMVβgal 1×10^{10} pfu/ml, sacrificed at 7 days, post-fixed in 4% paraformaldehyde, sectioned at 40 μm, and stained in X-gal solution. (A) Micrograph of control cervical spinal cord at low power ($50 \times$). (B) β-gal staining in a virus-injected animal at the same level and magnification. (C) Motor neurons of virally injected animals ($200 \times$). (D) Dorsal horn of viral-injected animal ($200 \times$); note the fiber plexus. (E) Immunocytochemistry of viral animals injected, preserved as above, and sectioned at 30 μm and processed with a β-gal antibody (5-Prime 3-Prime) ($200 \times$). (F) Control tissue ($200 \times$).

paramedian fashion, the distribution of labeled cells was bilateral with no obvious indication of the injected side. The sections shown were 0.5 cm from the injection site. Sections closer to the injection site show nearly all cells expressing enzyme activity while sections taken from 1.0 cm have a reduced fraction of cells stained (data not shown). In some animals, we observed labeling in dorsal roots (Fig. 3D) and neurons in the corresponding dorsal-root ganglion (not shown). Analysis of β-gal-expressing cells using immunocytochemistry revealed a similar pattern of cellular labeling as the histochemical reaction (Fig. 3E–F).

4. Discussion

The present study compares two techniques for delivering replication deficient adenovirus into rat spinal cord: intrathecal and intraparenchymal injections. Parenchymal injections resulted in a high level of *LacZ* expression at the injection site in the cervical cord. Enzyme activity peaked at 7 days and remained elevated but at a decreased level at 14 days. Rats examined at 60 days still displayed enzyme activity which, while significant, was only slightly elevated above non-injected controls. Low levels of enzyme activity were seen as far as 3 cm from the injection site at 14 days. No evidence of rostral spread into the brain was seen using MUG assays of hypothalamus, thalamus, frontal cortex and midbrain (data not shown).

Histochemical and immunocytochemical analyses revealed a similar cellular distribution of β-gal enzyme within the spinal cord. Microscopic examination showed that all cell types in the ventral gray matter of the rat spinal cord were transfected by the viral particles although further work with glial- and neuronal-specific markers

would provide additional confirmation. Motor neurons, which are readily distinguishable, exhibited the most intense staining. This may be a function of their size (surface area for viral uptake), number of viral binding sites expressed, or the positioning of the needle in the ventral cord (greater concentration of virus). The diverse cellular tropism (neurons, glia) is consistent with previous work in brain tissue by other groups [1,4,17]. Our data also support the idea of viral uptake by nerve terminals and retrograde transport and expression of the inserted gene because in some animals, x-gal staining was observed in dorsal nerve roots and terminal arbors in the dorsal horn. This expression is consistent with initial axonal transport of the virus to the cell bodies of dorsal root ganglion (DRG) neurons and then export of the enzyme to the synaptic terminals. In fact, in the animals in which the dorsal rootlets were stained, β-gal activity was also demonstrable in DRG neurons. Retrograde transport of the virus has been previously described in substantia nigra following adenovirus injection into the striatum [1]. In our preparations, no staining was observed in the white matter [1,18].

In contrast to the robust increase in β-gal activity with intraparenchymal injections, intrathecal injections of the same amount of virus increased enzyme activity in the spinal-cord tissue to a level approximately 50% above blank. However, in terms of 'spatial' distribution, much more spinal-cord tissue was affected. Significant increases in enzyme activity were detected along the entire spinal cord with peak levels in segments nearest the injection site in the sacral region. The pia mater, forming the boundary of the cerebral spinal fluid, also displayed β-gal activity after intrathecal injection and had a much greater level of expression than the spinal-cord parenchyma. Several factors may be responsible for the higher level of viral uptake into the pia as compared to the spinal cord upon instillation of the virus into the CSF space. One possibility is that connective tissue cells of the pia and arachnoid membranes have a greater number of binding sites for the virus leading to enhanced viral uptake. A second possibility is that the pial membrane represents a barrier in transferring particulates from the CSF to the spinal cord. This barrier, formed by pia, basement membrane and glia completely surrounds the spinal cord (for reviews, see Refs. [20,22]. Small molecules are freely permeable across the pial–glial barrier (e.g., neuropeptides [7]). Adenovirus is approximately 70 nm $\times$ 90 nm in size [10] and is too large to penetrate this barrier and yet while in contact with the pial membrane, readily enters these connective tissue cells. Improved techniques in temporary disruption of the pia-glial barrier may enhance the uptake of viral particles in spinal-cord neurons. Thus, an intrathecal injection, a routine and less traumatic procedure, could be used to obtain a greater level of cellular transduction in spinal cord using adenoviral vectors.

A third technique for viral gene expression in spinal-cord neurons is peripheral injection of adenovirus into muscle [12,13]. The injected viral particles are retrogradely transported to corresponding motor neuron cell bodies in the ventral spinal cord. This has the advantage of transfecting motor neurons without breaching the protective meningeal barrier and potentially damaging the CNS (with a needle). However, the technique limits the viral expression exclusively to the motor neuron cell bodies innervating the muscle fibers and does not benefit from the potential immune protection of the CNS.

The duration of expression of the transfected gene is also an important determinant of how these vectors can be used. Adenovirus does not integrate into the host genome and it is not clear what regulates termination of enzyme activity. In this study, enzyme activity peaked at 7 days, was 17% of the peak value at 14 days and was still slightly above background at 60 days, a time course similar to that seen with a CMV promoter after injection into brain [1,17]. Expression from a RSV promoter was comparatively much shorter. Peak expression occurred at 4 days and was diminished at 6 days [2]. Data from a variety of studies suggest that the duration of expression is tissue, transgene and species dependent. For example, adenoviral-mediated β-gal expression was detectable for 14 days in mouse liver with a β-actin promoter and a CMV enhancer (AdCB*lacZ* [11]), for 3 days in rat liver with a RSV promoter (Adβgal [14]) and for several months in mouse skeletal muscle with a RSV promoter (Adβgal [26]). Thus, in spinal cord, other promoters or combinations of promoters might provide a more optimal construct. The issues of promoters and integration on degree and duration of expression were examined in two recent papers. In spinal cord, injections of adeno-associated virus (AAV) expressing green fluorescent protein as the reporter showed, using cell counts, that the most robust transduction was obtained with a neuron-specific enolase promoter as compared to platelet-derived growth factor promoter and that detectable expression was seen out to 2 to 3 months [21]. A replication defective vector based on the human immunodeficiency virus and capable of genomic integration appears to provide a more robust expression over long durations (out to 6 months) in comparison to adenovirus, AAV or a murine leukemia virus [4]. These data suggest that both promoter and integration capability can be manipulated for optimal transgene expression.

In assessing the practicality of transferring these techniques to clinical practice, a recent concern has been raised regarding the immune response to adenoviral vectors in the CNS. Injection of replication deficient adenovirus has been reported to generate a measurable host inflammatory response in the CNS consisting of a biphasic response at both the injection site and along retrograde pathways [27]. In these studies, the initial injection resulted in a minimal host response, while repeated exposure (even peripheral) resulted in a marked inflammatory response [5,6]. The inflammatory effects appear to be more evident when high concentrations of virus are injected. In our study, using the

Sprague–Dawley strain of rats, aside from the expected trauma associated with the needle, no evidence of inflammation or obvious behavioral changes (e.g., motor impairment) were noted. Current work with vectors in which all viral coding sequences have been eliminated may further minimize the host response [16].

In summary, gene transfer offers an opportunity to study and treat disease processes at the level of the spinal cord. This is the first study that directly compares intrathecal vs. intraparenchymal routes of administration of adenoviral-mediated gene transfer. Using quantitative methodology, we demonstrated that the pial glial membrane is profoundly effective in preventing viral entry into the spinal cord. However, direct injection into tissue is extremely effective. Thus, more work is needed to safely bypass the pial barrier to further increase the efficiency of intrathecal injections and decrease the invasiveness of gene transfer to the spinal cord in vivo.

Acknowledgements

This research was supported in part by NIH grant No. T32 GM07612.

References

[1] S. Akli, C. Caillaud, E. Vigne, L.D. Stratford-Perricaudet, L. Poenaru, M. Perricaudet, A. Kahn, M.R. Peschanski, Transfer of a foreign gene into the brain using adenovirus vectors, Nat. Genet. 3 (1993) 224–228.

[2] G. Bajocchi, S.H. Feldman, R.G. Crystal, A. Mastrangeli, Direct in vivo gene transfer to ependymal cells in the central nervous system using recombinant adenovirus vectors, Nat. Genet. 3 (1993) 229–234.

[3] T.C. Becker, R.J. Noel, W.S. Coats, A.M. Gomez-Foix, T. Alam, R.D. Gerard, C.B. Newgard, Use of recombinant adenovirus for metabolic engineering of mammalian cells, Meth. Cell. Biol. 43 (1994) 161–189, Pt A.

[4] U. Blomer, L. Naldini, T. Kafri, D. Trono, I.M. Verma, F.H. Gage, Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector, J. Virol. (1997) 664d1–6649.

[5] A.P. Byrnes, R.E. MacLaren, H.M. Charlton, Immunological instability of persistent adenovirus vectors in the brain: peripheral exposure to vector leads to renewed inflammation, reduced gene expression, and demyelination, J. Neurosci. 16 (1996) 3045–3055.

[6] A.P. Byrnes, J.E. Rusby, M.J. Wood, H.M. Charlton, Adenovirus gene transfer causes inflammation in the brain, Neuroscience 66 (1995) 1015–1024.

[7] R.M. Caudle, L. Isaac, A novel interaction between dynorphin(1-13) and an *N*-methyl-D-aspartate site, Brain Res. 443 (1988) 329–332.

[8] R.M. Chanock, W. Ludwig, R.J. Heubner, T.R. Cate, L.W. Chu, Immunization by selective infection with type 4 adenovirus grown in human diploid tissue cultures. I. Safety and lack of oncogenicity and tests for potency in volunteers, JAMA 195 (1966) 445–452.

[9] B.L. Davidson, E.D. Allen, K.F. Kozarsky, J.M. Wilson, B.J. Roessler, A model system for in vivo gene transfer into the central nervous system using an adenoviral vector, Nat. Genet. 3 (1993) 219–223.

[10] S.E. Doran, X.D. Ren, A.L. Betz, M.A. Pagel, E.A. Neuwelt, B.J. Roessler, B.L. Davidson, Gene expression from recombinant viral vectors in the central nervous system after blood–brain barrier disruption, Neurosurgery 36 (1995) 965–970.

[11] J.F. Engelhardt, X. Ye, B. Doranz, J.M. Wilson, Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 6196–6200.

[12] F. Finiels, R. Gimenez, M. Barkats, M.L. Samolyk, J.J. Robert, A. Privat, F. Revah, J. Mallet, Specific and efficient gene transfer strategy offers new potentialities for the treatment of motor neurone diseases, Neuroreport 7 (1995) 373–378.

[13] G.D. Ghadge, R.P. Roos, U.J. Kang, R. Wollmann, P.S. Fishman, A.M. Kalynych, E. Barr, J.M. Leiden, CNS gene delivery by retrograde transport of recombinant replication-defective adenoviruses, Gene Ther. 2 (1995) 132–137.

[14] H.A. Jaffe, C. Danel, G. Longenecker, M. Metzger, Y. Setoguchi, M.A. Rosenfeld, T.W. Gant, S.S. Thorgeirsson, L.D. Stratford-Perricaudet, M. Perricaudet, Adenovirus-mediated in vivo gene transfer and expression in normal rat liver, Nat. Genet. 1 (1992) 372–378.

[15] M.D. Kawaja, J. Ray, F.H. Gage, Employment of fibroblasts for gene transfer: applications for grafting into the central nervous system, Genet. Eng. 13 (1991) 205–220.

[16] S. Kochanek, P.R. Clemens, K. Mitani, H.H. Chen, S. Chan, C.T. Caskey, A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 5731–5736.

[17] L.G. La Salle, J.J. Robert, S. Berrard, V. Ridoux, L.D. Stratford-Perricaudet, M. Perricaudet, J. Mallet, An adenovirus vector for gene transfer into neurons and glia in the brain, Science 259 (1993) 988–990.

[18] F. Lisovoski, J. Cadusseau, S. Akli, C. Caillaud, E. Vigne, L. Poenaru, L. Stratford-Perricaudet, M. Pericaudet, A. Kahn, M. Peschanski, In vivo transfer of a marker gene to study motoneuronal development, Neuroreport 5 (1994) 1069–1072.

[19] G.R. MacGregor, G.P. Nolan, Fiering, M. Roederer, L.A. Herzenberg, Use of *E. coli LacZ* (β-galactosidase) as a reporter gene. In: E.J. Murray, J.M. Walker (Eds.), Gene Expression In Vivo, Humana Press, Clifton, NJ, 1989.

[20] J.W. Millen, D.H.M. Woollam, On the nature of the pia mater, Brain 84 (1961) 514–520.

[21] A. Peel, S. Zolotukhin, G.W. Schrimsher, N. Muzyczka, P.J. Reier, Efficient transduction of green fluorescent protein in spinal cord neurons using adeno-associated virus vectors containing cell type specific promoters, Gene Ther. 4 (1997) 16–24.

[22] A. Peters, S.L. Palay, H. Webster, The Fine Structure of the Nervous System, Oxford University Press, New York, 1991, pp. 395–406.

[23] Z. Sahenk, J. Seharaseyon, J.R. Mendell, A.H. Burghes, Gene delivery to spinal motor neurons, Brain Res. 606 (1993) 126–129.

[24] S. Shimohama, M.B. Rosenberg, A.M. Fagan, J.A. Wolff, M.P. Short, X.O. Breakefield, T. Friedmann, F.H. Gage, Grafting genetically modified cells into the rat brain: characteristics of *E. coli* beta-galactosidase as a reporter gene, Brain Res., Mol. Brain Res. 5 (1989) 271–278.

[25] A.E. Smith, Viral vectors in gene therapy, Annu. Rev. Microbiol. 49 (1995) 807–838.

[26] L.D. Stratford-Perricaudet, I. Makeh, M. Perricaudet, P. Briand, Widespread long-term gene transfer to mouse skeletal muscles and heart, J. Clin. Invest. 90 (1992) 626–630.

[27] M.J. Wood, H.M. Charlton, K.J. Wood, K. Kajiwara, A.P. Byrnes, Immune responses to adenovirus vectors in the nervous system, Trends Neurosci. 19 (1996) 497–501.

[28] T.L. Yaksh, T.A. Rudy, Chronic catheterization of the spinal subarachnoid space, Physiol. Behav. 17 (1976) 1031–1036.

[29] M. Zimmermann, Ethical guidelines for investigations of experimental pain in conscious animals, Pain 16 (1983) 109–110.